**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**In re the Application of:**
DARFLER *et al.*

**Serial No.:** 10/796,288

**Filed:** March 10, 2004

**For:** Liquid tissue preparation from histopathologically processed biological samples, tissues and cells

**Group Art Unit:** 1657

**Examiner:** Clark D. Petersen, Ph.D.

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**DECLARATION OF MS. MARLENE DARFLER UNDER 37 C.F.R. § 1.132**

I hereby declare the following:

1. I am Marlene Darfler, a citizen of the United States, and I reside at 7000 Needwood Road, Derwood, MD 20855.

2. I am currently the Vice President Product Development at Expression Pathology, Inc. in Gaithersburg, MD. My *Curriculum Vitae* is attached hereto

3. I have been engaged in research in the fields of molecular biology and protein analysis since 1972.

4. I am a co-inventor of the invention described in U.S. Patent Application No. 10/796,288 (hereinafter "the '288 Application").

5. I have reviewed the Office Action dated August 10, 2007, U.S. Patent No. 5,672,696 to Wang *et al.* (hereinafter "*Wang*") and the 1995 *BioTechniques* reference by Banerjee *et al.* (hereinafter "*Banerjee*") that were cited in that Office Action.

6. *Wang* describes methods of preparing paraffin-embedded biological samples for gene analysis or PCR amplification. (See *Wang* at Abstract.)

7. *Banerjee* describes a microwave-based method of extracting DNA from formaldehyde-fixed, paraffin-embedded tissue sections. (See *Banerjee* at p. 768, col. 3.)

9. The rejections in the Office Action appear to be based on the assumption that methods intended to be used in the isolation and analysis of DNA are also applicable in protein expression analyses. That assumption is not justifiable, as explained below.

10. Experimental analyses were performed to compare and contrast the methods described in the '288 Application with the methods taught by *Wang* and *Banerjee*. The methods described in the '288 Application are consistent with the protocol described in the Liquid Tissue MS Protein Prep Kit manual (Expression Pathology, Inc.). Formalin fixed paraffin embedded mouse liver tissue was used as the starting material in each protocol. The biomolecule lysate from the Liquid Tissue preparation is contained in a single tube and the entire tissue sample was rendered in a visually solubilized form. This lysate is not fractionated prior to protein analysis. Furthermore, the preparation remains in the same tube through the entire processing of the tissue sample.

11. The *Wang* preparation protocol was performed based on the parameters provided in *Wang*, particularly Example 1, which provides a DNA extraction method using 10% (weight

to volume) m-hydroxybenzoic acid. The biomolecule lysate resulting from the *Wang* protocol was fractionated, as directed in the protocol, into three separate tubes. The first fraction, the insoluble fraction, contained insoluble material consisting primarily of visually insoluble tissue and could not be analyzed for proteins because of the insolubility of the material. The second fraction, the visually soluble liquid fraction, was not further fractionated prior to protein analysis. The third fraction, the DNA fraction, was rendered insoluble by a precipitation step and was subsequently resuspended into a visually solubilized solution according to the protocol prior to protein analysis.

12. The *Banerjee* preparation was generated using the protocol described in the reference at pages 770-72. The biomolecule lysate resulting from the *Banerjee* protocol was fractionated, as directed in the protocol, into two separate tubes. The first fraction, the insoluble fraction, contained visually insoluble material and could not be analyzed for proteins because of the insolubility of the material. The second fraction, the visually soluble liquid fraction, was not further fractionated prior to protein analysis.

13. Proteins were analyzed in each of the biomolecule lysates utilizing mass spectrometry (MS). MS was utilized because this technology is capable of identifying thousands of individual peptides and proteins in a single analysis, thereby providing an overall representation of protein expression in a biomolecule lysate. The presence of DNA in the *Wang* and *Banerjee* preparations was analyzed by one-dimensional electrophoresis in a 1% agarose gel prepared by use of 1X TAE buffer and containing 0.5 μg/ml of ethidium bromide. Fluorometric detection of DNA was carried out by means of a UV illuminator after electrophoresis. Results were compared to a DNA size standard.

14. All the proteins identified by the MS analysis of the four samples were further analyzed using protein analysis software from the Gene Ontology website (GO) (geneontology.org) in order to determine their biological and molecular functions. The GO analysis indicate the types and number of proteins involved in liver function that were identified in each of the biomolecule lysates. Because these biomolecule lysates were made from histopathologically processed mouse liver tissue, the presence of identified proteins that are involved in normal liver function is helpful to demonstrate representation of protein expression reflecting liver tissue origin.

15. A 2µl aliquot of the biomolecule lysate resulting from the Liquid Tissue protocol, in completely soluble liquid form, was directly injected to the MS instrument without any prior fractionation. The mass spectrometer was operated under standard global protein analysis parameters as utilized for each of the four biomolecule lysates in this report. Results as shown in Figure 1 indicate positive identification of 1,251 different, unique proteins in this biomolecule lysate.

16. A 2µl aliquot of the liquid fraction resulting from the *Wang* protocol was injected directly into the MS instrument without any further fractionation. The mass spectrometer was operated under standard global protein analysis parameters as utilized for each of the four biomolecule lysates in this report. Results as shown in Figure 1 indicate positive identification of 107 different, unique proteins in this biomolecule lysate. This number of positively identified proteins is only 15% of that identified in the Liquid Tissue lysate.

17. A 2µl aliquot of the resuspended DNA fraction resulting from the *Wang* protocol was injected directly into the MS instrument without any further fractionation. The mass

spectrometer was operated under standard global protein analysis parameters as utilized for each of the four biomolecule lysates in this report. Results as shown in Figure 1 indicate positive identification of 12 different, unique proteins in this biomolecule lysate. This number of positively identified proteins is only 0.96% of that identified in the Liquid Tissue lysate.

18. A 2μl aliquot of the biomolecule lysate resulting from the *Banerjee* protocol was injected directly into the MS instrument without any further fractionation. The mass spectrometer was operated under standard global protein analysis parameters as utilized for each of the four biomolecule lysates in this report. Results as shown in Figure 1 indicate positive identification of 15 different, unique proteins in this biomolecule lysate. This number of positively identified proteins is only 1.2% of that identified in the Liquid Tissue lysate.

19. In my opinion, these results indicate that a much larger number of proteins are identified from a single Liquid Tissue preparation than from the other three preparations. The total number of proteins from the Liquid Tissue preparation can be utilized in a global bioinformatic profile to determine the types of proteins expressed in order to determine representational protein expression of the starting material from a single preparation. The other three preparations cannot provide enough protein expression information for a similar analysis. In fact, analyzable protein from the *Wang* preparation is found in two completely separate fractions, not a single soluble lysate, and thus any lysate prepared utilizing the *Wang* protocol cannot claim protein expression that is representational of the starting biological sample.

20. Analyses were performed using the Gene Ontology function for cellular component representation. GO analysis of the Liquid Tissue lysate demonstrates that this preparation provides for the identification of proteins that represent a wide range originating

from 124 different regions across every part of the cell. In contrast, GO analysis of the liquid fraction from the *Wang* lysate indicates that the 107 proteins identified in this lysate originate from only 30 different regions within the cell, while GO analysis of the DNA fraction from the *Wang* lysate indicates that the 12 proteins identified in this lysate originate from only 8 different regions within the cell. This result also demonstrates that a subset of the protein in the starting material from the *Wang* preparation ends up in the DNA fraction, separate from the liquid fraction. GO analysis of the *Banerjee* preparation indicates that of the 15 proteins identified in this lysate, only 8 originate in different regions within the cell.

21. Analyses were also performed to identify those proteins present in each biomolecule lysate that are involved in liver function, which is in my opinion a strong additional indicator of representation of the total protein content of the histopathologically processed biological sample. A total of 677 proteins of the 1,251 proteins identified in the Liquid Tissue lysate are involved in normal liver function. In marked contrast, there were 10 liver function proteins identified in the *Wang* liquid fraction lysate and 3 in the *Wang* DNA fraction lysate; there were 2 liver function proteins identified in the *Banerjee* lysate.

22. In my opinion, there are four standard proteins that are produced by the liver and whose presence is assayed for in the blood for this widely-applied clinical assay: alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase, and albumin. Evaluation of the protein expression data across all four of the lysates we tested indicates that all of these proteins were identified as expressed in the Liquid Tissue lysate while none of these proteins was identified as expressed in the other three analyzed lysates.

23. It is my opinion that the cumulative results from analyses of these data indicate that a majority of the proteins from the Liquid Tissue lysate are involved in the biochemical and biological functions of normal liver. Thus, the Liquid Tissue lysate is representative of the starting material originating from a histopathologically processed liver sample. Furthermore, the lack of liver function proteins identified in the other three preparations indicates that these lysates are not representative of the starting material originating from a histopathologically processed liver sample.

24. Because the *Wang* and *Banerjee* lysates are physically fractionated and much of the protein content cannot be analyzed for representative protein expression of mouse liver, these methods do not result in samples that are representative of the total protein content of the tissue sample.

25. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code and may jeopardize the validity or enforceability of the above-identified application or any patent issued thereon.

Marlene M Darfler — 10-31-07

Marlene Darfler — Date

**Marlene M. Darfler**
7000 Needwood Road
Derwood, MD 20855

Biotechnology professional concentrating in the area of molecular and cellular biology product development. Extensive experience in development and technology transfer in both the diagnostics and life science research products markets. Instrumental in building Expression Pathology, Inc., both scientifically and operationally.

**Professional Experience**

| | |
|---|---|
| Expression Pathology, Inc. Gaithersburg, MD | |
| Co-Founder, Vice President Product Development | 2001 - Present |
| Invitrogen (formerly Life Technologies Inc.) | |
| Molecular and Cellular Biology R&D Rockville, MD | |
| Staff Scientist, Amplification Group | 1997 - 2001 |
| Staff Scientist, Cell Biology Group | 1994 - 1997 |
| Staff Scientist, Genome Analysis Group | 1990 - 1994 |
| Life Technologies, Inc. | |
| Diagnostics Division Rockville, MD | |
| Scientist, Diagnostics Product Development | 1986 - 1990 |
| Life Technologies, Inc. | |
| Corporate R&D Rockville, MD | |
| Biochemist, Immunology Product Development | 1983 - 1986 |
| Hybritech Incorporated San Diego, CA | |
| Research Associate, Immunoassay Product Development | 1981 - 1982 |
| Veterans Administration Hospital San Diego, CA | |
| Medical Technologist / Pharmacokinetics Research Associate | 1979 - 1981 |
| University of Rochester Strong Memorial Hospital Rochester, NY | |
| Senior Medical Technologist / Research Assistant | 1972 – 1979 |

**Education**

| | |
|---|---|
| Masters in Engineering Management | George Washington University, Washington, DC |
| MT (ASCP) Medical Technology | Albany Medical College, Albany, NY |
| BS Biology | State University of New York at Albany, Albany, NY |

**Professional Memberships:**

2004 - Member, Association for Molecular Pathology
2004 - Member, Women in Bio
2006 - Member, American Chemical Society
2007 - Member, American Society for Investigative Pathology

**Research Publications:**


1. Hood, B., Darfler, M., Guiel, T., Furusato, B., Lucas, D., Ringeisen, B., Sesterhenn, I., Conrads, T., Veenstra, T. and Krizman, D. (2005). Proteomic analysis of formalin-fixed prostate cancer tissue. Molecular and Cellular Proteomics, 4 (11): 1741-1753.

2. Barron, J., Young, H., Dlott, D., Darfler, M., Krizman, D. and Ringeisen, B. (2005). Printing of protein microarrays via a capillary-free fluid jetting mechanism. Proteomics, 5(16):4138-44.

3. DaRue A. Prieto, D., Hood, B., Darfler, M., Guiel, T., Lucas, D., Conrads, T., Veenstra, T. and Krizman, D. (2005). Liquid Tissue™: proteomic profiling of formalin-fixed tissues. BioTechniques, 38:S32-S35.

4. Nazarenko, I., Lowe, B., Darfler, M., Ikonomi, P., Schuster, D., and Rashtchian, A.. (2002). Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. Nucleic Acids Research, 30(9): e37.

5. Richie, K., Goldsborough, M., Darfler, M., Benzinger, E.A., Lovekamp, M.L., Reeder, D. and C. O'Connell, C. (1999). Long PCR for VNTR Analysis. Journal of Forensic Sciences, 44(6):1176-85.

6. Magnani, I., Meneveri, R., Marozzi, A., Ginelli, E., Fuhrman-Conti, A.M., Darfler, M., Monard, S.P. and Sacchi, N. (1997). Functional marker chromosomes lacking detectable alpha satellite DNA: a tool for centromere study. Chromosome Research, 6, 69-71.

7. Sacchi, N., Schiaffonati, L., Magnani, I., Pappalardo, C., Hughes, A.J., Darfler, M. and Hoogeveen, A.T. (1996). Detection and subcellular localization of an AML1 chimeric protein in t(8;21) positive acute myeloid leukemia. Oncogene, 12, 437-444.

8. Sacchi, N., Magnani, I. Fuhrman-Conti, A.M., Monard, S.P., and Darfler, M. (1996). A stable marker chromosome with a cryptic centromere: evidence for centromeric sequences associated with an inverted duplication. Cytogenet Cell Genet, 73:123-129.

9. Sacchi, N., Magnani, I., Kearney, L., Wijsman, J., Hagemeijer, A., and Darfler, M. (1995). Interphase cytogenetics of the t(8;21)(q22;q22) associated with acute myelogenous leukemia by two-color fluorescence *in situ* hybridization. Cancer Genet Cytogenet, 79, 97-103.

10. Magnani, I., Sacchi, N., Darfler, M., Nisson, P.E., Tornaghi, R., Fuhrman-Conti, A.M. (1993). Identification of the chromosome 14 origin of a C-negative marker associated with a 14q32 deletion by chromosome painting. Clin Genet 43, 180-185.

11. Mackey, J., Darfler, M., Nisson, P., and Rashtchian, A. (1993). Use of random primer extension for concurrent amplification and nonradioactive labeling of nucleic acids. Analytical Biochemistry, 21, 428-435.

12. Nuovo, G.J., Darfler, M.M., Impraim, C.C., Bromley, S.E. (1991). Occurrence of multiple types of human papillomavirus in genital tract lesions, analysis by *in situ* hybridization and polymerase chain reaction. American Journal of Pathology 138:1, 53-58.

13. Bromley, S.E. and Darfler, M.M. (1990). "Hybridization *in situ*: application in the detection of human papillomavirus," Papillomaviruses in Human Pathology Recent Progress in Epidermoid Precancers, Serono Symposia Publications, vol 78, New York: Raven Press, 475.

14. Bromley, S.E., Darfler, M.M., Hammer, M.L., Jones-Trower, A., Primus, M.A., Kreider, J.W. (1990) "*in situ* hybridization to human papillomavirus DNA in fixed tissue samples: comparison of detection methods." Papillomaviruses, Wiley-Liss, Inc., 35.

**Industry Publications:**

1. Schuster, D.M., Darfler, M., Lee, J.E. and Rashtchian, A. (1998). Improved sensitivity and specificity of RT-PCR. Focus, 20, 34-35.

2. Westfall, B., Darfler, M., Solus, J., Xu, R. and Rashtchian, A. (1998). Biochemical characterization of Platinum *Taq* DNA polymerase. Focus, 20, 1718.

3. El-Badry, O.M. and Darfler, M.M. (1996). Evaluation of biotinylated oligonucleotide probes for *in situ* hybridization. Focus, 18, 70-72.

4. Darfler, M., Dougherty, C. and Goldsborough, M. (1996). The Mouse Y•ES System: a novel reagent system for the evaluation of mouse chromosomes. Focus, 18, 15-16.

5. Darfler, M.M., Karaszkiewicz, J.W. (1995). In Situ Localization of Apoptosis Using Terminal Deoxynucleotidyl Transferase. Focus 17:3, 81.

6. Nisson, P.E., Darfler, M.M., and Watkins, P.C. (1994). Cosmid fingerprinting and somatic cell hybrid characterization using biotinylated human COT-1 DNA. Focus 16:1, 26.

7. Darfler, M.M., Nisson, P.E., Watkins, P.C. (1992). Painting human chromosomes by *in situ* hybridization: novel reagent systems for chromosome detection and analysis. Focus 14:2, 58.

Table 1

# Method
## Liquid Tissue Preparation

**Purpose:**

- Method according to EPI patent application.
- Reference sample.

**Steps:**

- Use the protocol as stated in EPI's Liquid Tissue MS Protein Prep Kit manual.
- Place a tissue section (10 um thick) on a microscope slide.
- Heat the slide at 60°C for 30 minutes to melt the paraffin.
- Place the slide in a container of SubX for 5 minutes.
- Transfer the slide to a fresh container of SubX for 5 minutes.
- Transfer the slide to a container of 100% ethanol for 5 minutes.
- Transfer the slide to a fresh container of 100% ethanol for 5 minutes.
- Transfer the slide to a container of 85% ethanol for 1 minute.
- Transfer the slide to a container of 70% ethanol for 1 minute.
- Transfer the slide to a container of high-purity water for 1 minute.
- Pipet 20 ul of Liquid Tissue Buffer into a 1.5 ml tube.
- Collect ~30,000 cells from the deparaffinized tissue section (8 $mm^2$ area) by scraping with a needle and place the tissue in the tube containing the buffer.
- Heat the tube in a heating block at 95°C for 90 minutes.
- Every 20 minutes, remove the tube from the heating block and shake down the buffer so that it covers the cells by flicking the tube in a downward motion. Immediately place the tube back into the heating block. DO NOT allow the tube to cool down completely.
- After 90 minutes at 95°C, microcentrifuge the tube at 10,000 rcf for 1 minute.
- Cool the tube on ice for 1 to 2 minutes.
- Reconstitute the lyophilized Trypsin with 20 µl of Trypsin Diluent.
- Add 1.0 µl of the Trypsin Solution to the tube.
- Mix and briefly microcentrifuge to collect the solution at the bottom of the tube.
- Heat the tube in a waterbath at 37°C. For the first hour, every 20 minutes, remove the tube, and vortex rigorously for 10 to 15 seconds. Shake the buffer down to the bottom of the tube so that it covers the cells before placing the tube back into the waterbath.
- Continue incubating at 37°C overnight (16 to 18 hours).
- At the end of the 37°C incubation, microcentrifuge the tube at 10,000 rcf for 1 minute.
- Add 2 µl of 100 mM DTT. Mix and briefly microcentrifuge to collect the solution at the bottom of the tube.
- Heat the tube at 95°C for 5 minutes.
- Microcentrifuge the tube at 10,000 rcf for 1 minute. **Figure 1.**
- Analyze by mass spectrometry.

Table 2

# Method
## Wang Preparation

**Purpose:**

- Method according to Wang patent (5,672,696) Claim #1.
- Demonstrate that this preparation is not representative of the total protein content of the tissue sample.

**Steps:**

- Use the preferable conditions as stated in Wang patent specifications.
- Place tissue section (equivalent of 10 um thick, 10 mm times 20 mm area) in a 1.5 ml tube.
- Add 1 ml of SubX and mix for 3 minutes.
- Centrifuge at 12,000 rpm for 3 minutes at room temperature.
- Discard supernatant.
- Repeat addition of SubX, centrifuge and discard supernatant.
- Add 1 ml ethanol and mix for 3 minutes.
- Centrifuge 12,000 rpm for 3 minutes at room temperature.
- Discard supernatant.
- Repeat addition of ethanol, centrifuge and discard supernatant.
- Dry pellet.
- Add to the pellet 180 ul of 11 mM Tris, pH 7.0; 5.6 mM EDTA; 1.11% SDS.
- Heat at 90°C for 10 minutes.
- Centrifuge at 10,000 rpm for 5 minutes at room temperature.
- Add 20 ul of 1M DTT.
- Add 10 ul of 20 mg/ml papain (Sigma Cat # P4762).
- Incubate at 50°C for 90 minutes.
- Add 200 ul of 40% aqueous isopropanol, pH 6.0 containing 10% hydroxybenzoic acid (Sigma Cat # 54610 or H20008)
- Incubate at room temperature for 30 minutes.
- Centrifuge at 12,000 rpm for 5 minutes. **Figure 2 – Wang Fraction #1**
- Transfer supernatant to another tube.
- Add to the supernatant 40 ul of 3M NaCl and 900 ul of isopropanol.
- Incubate at room temperature for 10 minutes.
- Centrifuge at 12,000 rpm for 15 minutes.
- Transfer supernatant to another tube. **Figure 2 – Wang Fraction #2**
- Analyze supernatant by mass spectrometry.
- Add 1 ml of 70% ethanol to the precipitate and stir.
- Centrifuge at 15,000 rpm for 15 minutes at 4°C.
- Discard supernatant.
- Dry precipitate. **Figure 2 – Wang Fraction #3**
- Redissolve the precipitate in 20 ul of 10mM Tris, 1mM EDTA, pH 8.0.
- Analyze redissolved pellet by mass spectrometry.
- Analyze redissolved pellet by fluorometric detection of DNA carried out by means of a UV illuminator after electrophoresis.

Table 3

# Method
## Banerjee Preparation

**Purpose:**

- Method according to Banerjee publication (Biotechniques (1995) Vol. 18, No. 5: pp 768 -772).
- Demonstrate that this preparation is not representative of the total protein content of the tissue sample.

**Steps:**

- Place tissue section (equivalent of two 5 um thick sections) in a 1.5 ml tube.
- Add 200 ul of 50mM Tris-HCl, pH 8.5; 1mM EDTA; 0.5% Tween® 20.
- Microwave at 500 watts for a total of 30 seconds, in 15 second segments.
- Centrifuge at 12,000 rpm for 10 minutes.
- Remove and discard the paraffin ring.
- Resuspend the tissue pellet in the buffer by gentle shaking.
- Add 2.4 ul of 16.5 mg/ml (final concentration 200 ug/ml) Proteinase K (Sigma Cat # P2308).
- Incubate at 42°C overnight.
- Centrifuge for 5 minutes at 6000 rpm.
- Transfer supernatant to another tube. **Figure 3 – Banerjee Fraction #1 and Fraction #2**
- Boil supernatant for 10 minutes.
- Analyze supernatant by mass spectrometry.
- Analyze supernatant by fluorometric detection of DNA carried out by means of a UV illuminator after electrophoresis.

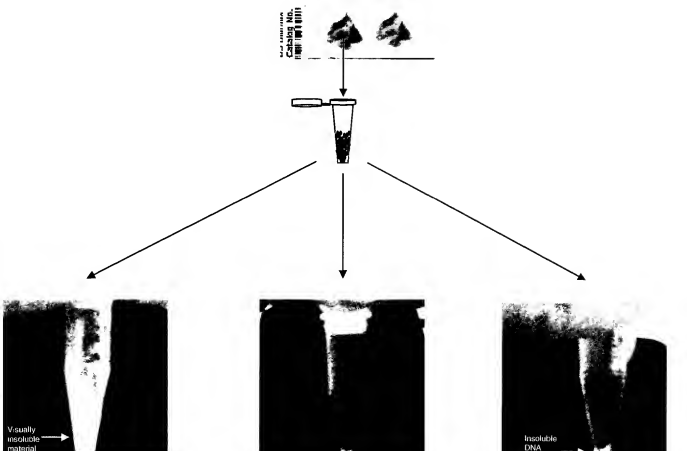
Figure 1

# Liquid Tissue Preparation


**Liquid Tissue lysate at the end of the protocol**

Figure 2

# Wang Preparation




**Wang Fraction #1**

**Wang Fraction #2**
**Liquid Fraction**

**Wang Fraction #3**
**DNA Pellet**

Figure 3

# Banerjee Preparation

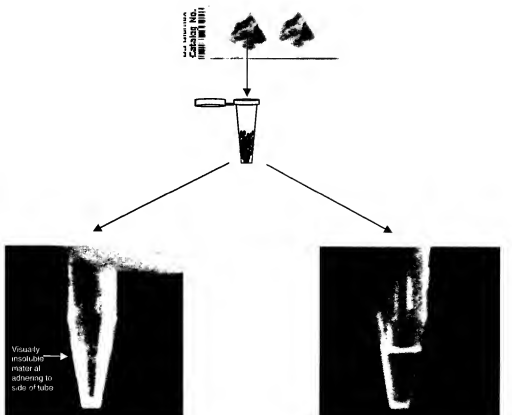
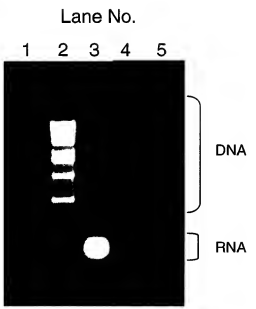


Figure 4

# DNA Analysis

Fluorometric detection of DNA carried out by means of a UV illuminator after electrophoresis.

1% Agarose Gel
1x TAE Buffer
0.5 ug/ml Ethdium Bromide




1 Gel Loading Buffer
2 1Kb DNA Ladder
3 Wang Preparation – Fraction #3
4 Banerjee Preparation – Fraction #2
5 Empty

Figure 5

# Method Comparison

## Total Number of Proteins Identified per Biomolecule Preparation

| | Liquid Tissue Prep | Wang Prep Liquid Fraction | Wang Prep DNA Fraction | Banerjee Prep |
|---|---|---|---|---|
| Total Number of Proteins Identified By Mass Spectrometry | 1,251 | 107 | 12 | 15 |

Figure 6

# Gene Ontology Analysis

## Liquid Tissue Preparation

### Cellular Component

1,251 proteins

- mitochondrial matrix
- cell junction
- nuclear envelope-endoplasmic reticulum
- chloroplast
- intermediate filament cytoskeleton
- apicolateral plasma membrane
- voltage-gated potassium channel complex
- endoplasmic reticulum membrane
- organellar ribosome
- nucleus
- intermediate filament
- nucleosome remodeling complex
- mitochondrial ribosome
- mitochondrial outer membrane
- collagen type IV
- transcription factor complex
- extracellular space
- basement membrane
- large ribosomal subunit
- endosome
- intercellular junction
- plastid
- microsome
- ribosome
- mitochondrial electron
- transport
- ribonucleoprotein complex
- inner membrane
- peroxisome
- microtubule associated complex
- outer membrane
- microbody
- cytosol
- cellular_component unknown
- extracellular
- DNA-directed RNA polymerase II
- apical plasma membrane
- nucleosome
- actin cytoskeleton
- respiratory chain complex I
- cell fraction
- cytoskeleton
- external encapsulating structure
- axonemal dynein complex
- tight junction
- pronucleus
- cell-cell adherens junction
- eukaryotic translation complex
- collagen type VII
- lamellipodium
- cell envelope
- myofibril
- periplasmic space
- collagen type IX
- lysosome
- ubiquinol-cytochrome-c complex
- respiratory chain complex III
- repairosome
- cytoplasm
- plasma membrane
- Arp2/3 protein complex
- ribulose bisphosphate complex
- adherens junction
- Golgi membrane
- mediator complex
- respiratory chain complex IV
- dynein complex
- membrane
- vesicular fraction
- membrane fraction
- chromatin
- sodium/potassium ATPase complex
- vacuole
- sarcomere
- periplasmic space
- respiratory chain complex III
- cytoplasmic vesicle
- extrachromosomal circular DNA
- alpha-glucosidase complex
- nuclear membrane
- facit collagen
- centromere
- amyloplast
- mitochondrial large ribosomal subunit
- stress fibers
- plastid stroma
- secretory granule
- microtubule cytoskeleton
- mitochondrial outer membrane
- extrachromosomal DNA
- Golgi trans face
- myosin
- lytic vacuole
- microtubule
- Golgi stack
- Golgi apparatus
- cilium
- integral to Golgi membrane
- cell projection
- organellar large ribosomal subunit
- NADH dehydrogenase complex
- kinesin complex
- nucleoplasm
- muscle fiber
- late endosome
- early endosome
- mitochondrion
- endoplasmic reticulum
- zonula adherens
- integral to membrane
- cell
- unlocalized
- collagen
- axoneme
- mitochondrial inner membrane
- endomembrane system
- integral to endoplasmic reticulum
- chloroplast stroma
- nucleotide excision repair complex
- intracellular
- integral to plasma membrane
- mitochondrial membrane
- respiratory chain complex I
- extracellular matrix

Figure 7

# Gene Ontology Analysis

## Wang Preparation – Liquid Fraction

### Cellular Component

107 Proteins

- cytoplasm
- intracellular
- mitochondrion
- COPII-coated vesicle
- obsolete cellular component
- cytoskeleton
- microtubule associated complex
- extracellular matrix
- respiratory chain complex IV
- clathrin-coated vesicle
- extracellular space
- microtubule cytoskeleton
- membrane
- integral to membrane
- cell
- chromatin
- collagen type VII
- collagen
- cytosol
- synaptic vesicle
- mitochondrial inner membrane
- nucleus
- inner membrane
- cytoplasmic vesicle
- extracellular
- respiratory chain complex IV
- mitochondrial electron transport
- coated vesicle
- chromosome
- mitochondrial membrane

Figure 8

# Gene Ontology Analysis

## Wang Preparation - DNA Fraction

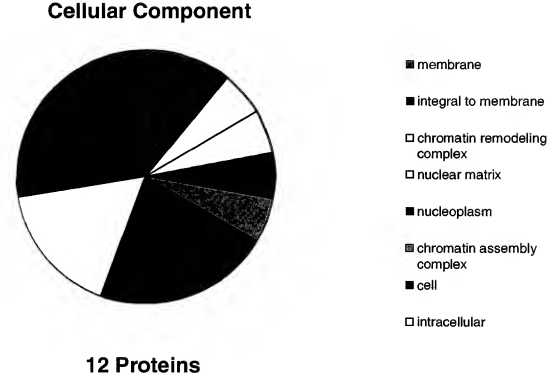


Figure 9

# Gene Ontology Analysis

## Banerjee Preparation

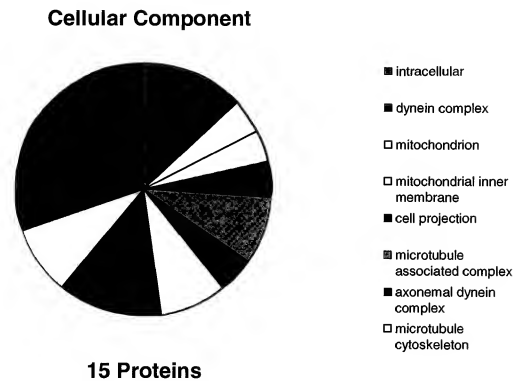


Figure 10

# Method Comparison

## Gene Ontology – Cellular Compartment

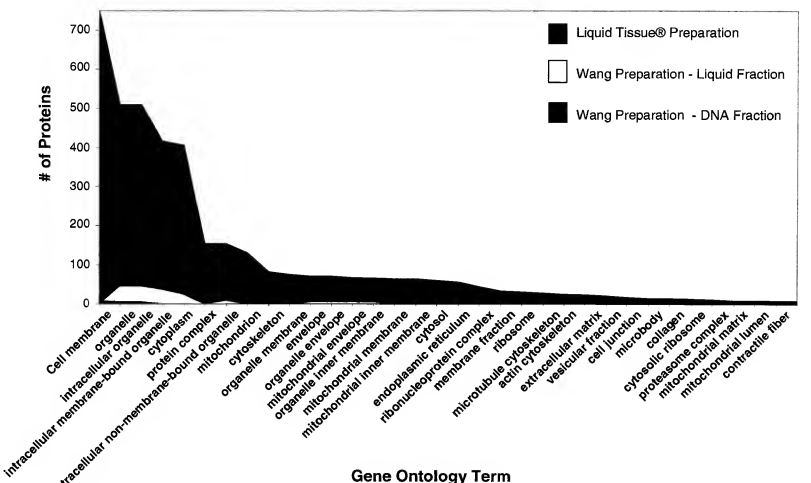


Table 4

# Method Comparison

## Representative Expression of Liver Function Proteins

| | Liquid Tissue Prep | Wang Prep Liquid Fraction | Wang Prep DNA Fraction | Banerjee Prep |
|---|---|---|---|---|
| **Glucose Utilization** | | | | |
| glucose metabolism | 5 | | | |
| glucose homeostasis | 1 | | | |
| cell glucose homeostasis | 1 | | | |
| UTP-glucose-1-phosphate activity | 1 | | | |
| **Glyogen Production** | | | | |
| glycogen biosynthesis | 2 | | | |
| glycogen metabolism | 2 | | | |
| glycogen (starch) synthase activity | 1 | | | |
| **Amino Acid Production** | | | | |
| amino acid and derivative metabolism | 12 | | | |
| amino acid activation | 4 | | | |
| aspartate family amino acid biosynthesis | 1 | | | |
| protein amino acid phosphorylation | 1 | | | |
| sulfur amino acid biosynthesis | 1 | | | |
| amino sugar metabolism | 1 | | | |
| protein amino acid sulfation | 1 | | | |
| protein amino acid glycosylation | 1 | | | |
| amino acid metabolism | 10 | | | |
| aspartate family amino acid metabolism | 1 | | | |
| serine family amino acid biosynthesis | 1 | | | |
| amino acid derivative catabolism | 1 | | | |
| serine family amino acid metabolism | 2 | | | |
| amino acid derivative metabolism | 2 | | | |
| amino acid biosynthesis | 2 | | | |
| sulfur amino acid metabolism | 2 | | | |
| acylaminoacyl-peptidase activity | 1 | | | |
| aminoacylase activity | 1 | | | |
| aminomethyltransferase activity | 1 | | | |

Table 5

# Method Comparison

## Representative Expression of Liver Function Proteins

| Lipid Metabolism and Production | Liquid Tissue Prep | Wang Prep Liquid Fraction | Wang Prep DNA Fraction | Banerjee Prep |
|---|---|---|---|---|
| lipid biosynthesis | 3 | | | |
| membrane lipid metabolism | 1 | | | |
| protein lipidation | 1 | | | |
| phospholipid transfer to membrane | 1 | | | |
| lipid metabolism | 10 | | | |
| phospholipid metabolism | 1 | | | |
| glycerolipid metabolism | 1 | | | |
| neutral lipid metabolism | 1 | | | |
| lipid transport | 2 | | | |
| lipid transporter activity | 2 | | | |
| phospholipid transporter activity | 1 | | | |
| phospholipid binding | 1 | | | |
| calcium-dependent phospholipid binding | 1 | | | |
| lipid binding | 1 | | | |

Table 6

# Method Comparison

## Representative Expression of Liver Function Proteins

| Protein Production | Liquid Tissue Prep | Wang Prep Liquid Fraction | Wang Prep DNA Fraction | Banerjee Prep |
|---|---|---|---|---|
| protein modification | 6 | | | |
| protein biosynthesis | 12 | | | |
| protein catabolism | 9 | | | |
| protein lipidation | 1 | | | |
| glycoprotein metabolism | 1 | | | |
| protein-disulfide reduction | 1 | | | |
| prenylated protein catabolism | 1 | | | |
| protein transport | 7 | | | |
| protein amino acid phosphorylation | 1 | | | |
| protein prenylation | 1 | | | |
| protein amino acid sulfation | 1 | | | |
| protein amino acid glycosylation | 1 | | | |
| regulation of protein catabolism | 1 | | | |
| glycoprotein biosynthesis | 1 | | | |
| lipoprotein biosynthesis | 1 | | | |
| lipoprotein metabolism | 1 | | | |
| negative regulation of protein catabolism | 1 | | | |
| protein targeting | 4 | | | |
| protein metabolism | 30 | 2 | | 1 |
| protein folding | 2 | | | |
| ribonucleoprotein complex | 5 | | | |
| Arp2/3 protein complex | 1 | | | |
| protein binding\, bridging | 1 | | | |
| protein binding | 25 | | 1 | |
| protein transporter activity | 1 | 1 | | |
| protein prenyltransferase activity | 2 | | | |
| protein homodimerization activity | 1 | | | |
| protein self binding | 1 | | | |
| protein disulfide oxidoreductase activity | 1 | | | |

Table 7

# Method Comparison

## Representative Expression of Liver Function Proteins

| General Metabolism | Liquid Tissue Prep | Wang Prep Liquid Fraction | Wang Prep DNA Fraction | Banerjee Prep |
|---|---|---|---|---|
| main pathways of carbohydrate metabolism | 6 | | | |
| nucleotide and nucleic acid metabolism | 35 | 1 | 1 | |
| energy reserve metabolism | 3 | | | |
| amine metabolism | 12 | | | |
| hexose metabolism | 3 | | | |
| amino acid and derivative metabolism | 12 | | | |
| water-soluble vitamin metabolism | 3 | | | |
| phosphate metabolism | 3 | | | |
| glucose metabolism | 3 | | | |
| vitamin metabolism | 3 | | | |
| phosphorus metabolism | 3 | | | |
| organic acid metabolism | 19 | | | |
| membrane lipid metabolism | 1 | | | |
| N-acetylneuraminate metabolism | 1 | | | |
| glycoprotein metabolism | 1 | | | |
| lipopolysaccharide metabolism | 1 | | | |
| glycine metabolism | 1 | | | |
| prenylcysteine metabolism | 1 | | | |
| icosanoid metabolism | 1 | | | |
| alcohol metabolism | 4 | | | |
| coenzyme A metabolism | 1 | | | |
| lipid metabolism | 10 | | | |
| phospholipid metabolism | 1 | | | |
| negative regulation of metabolism | 1 | | | |
| nucleotide-sugar metabolism | 1 | | | |
| amino sugar metabolism | 1 | | | |
| acyl-CoA metabolism | 1 | | | |
| NADPH metabolism | 1 | | | |
| biotin metabolism | 1 | | | |

Table 8

# Method Comparison

## Representative Expression of Liver Function Proteins

| General Metabolism (cont.) | Liquid Tissue Prep | Wang Prep Liquid Fraction | Wang Prep DNA Fraction | Banerjee Prep |
|---|---|---|---|---|
| pigment metabolism | 1 | | | |
| carboxylic acid metabolism | 19 | | | |
| methionine metabolism | 1 | | | |
| nicotinamide metabolism | 1 | | | |
| glycerolipid metabolism | 1 | | | |
| vitamin B6 metabolism | 1 | | | |
| pyridine nucleotide metabolism | 1 | | | |
| hormone metabolism | 1 | | | |
| acetate metabolism | 1 | | | |
| glycerol ether metabolism | 1 | | | |
| fatty acid metabolism | 4 | | | |
| acetyl-CoA metabolism | 1 | | | |
| aromatic compound metabolism | 1 | | | |
| bile acid metabolism | 1 | | | |
| citrate metabolism | 1 | | | |
| prostanoid metabolism | 1 | | | |
| isoprenoid metabolism | 1 | | | |
| lipoprotein metabolism | 1 | | | |
| thyroid hormone metabolism | 1 | | | |
| regulation of metabolism | 1 | | | |
| polyol metabolism | 1 | | | |
| amino acid metabolism | 10 | | | |
| metabolism | 113 | 6 | 1 | 1 |
| neutral lipid metabolism | 1 | | | |
| aspartate family amino acid metabolism | 1 | | | |
| porphyrin metabolism | 1 | | | |
| coenzyme metabolism | 10 | | | |
| prostaglandin metabolism | 1 | | | |
| oxidoreduction coenzyme metabolism | 1 | | | |

Table 9

# Method Comparison

## Representative Expression of Liver Function Proteins

| General Metabolism (cont.) | Liquid Tissue Prep | Wang Prep Liquid Fraction | Wang Prep DNA Fraction | Banerjee Prep |
|---|---|---|---|---|
| acylglycerol metabolism | 1 | | | |
| triacylglycerol metabolism | 1 | | | |
| biogenic amine metabolism | 1 | | | |
| coenzyme and prosthetic group metabolism | 11 | | | |
| glycerol metabolism | 1 | | | |
| sulfur metabolism | 5 | | | |
| heme metabolism | 1 | | | |
| CMP-N-acetylneuraminate metabolism | 1 | | | |
| TCA intermediate metabolism | 1 | | | |
| pyridoxine metabolism | 1 | | | |
| ribonucleotide metabolism | 2 | | | |
| nucleotide metabolism | 2 | | | |
| glucan metabolism | 2 | | | |
| nitrogen metabolism | 2 | | | |
| serine family amino acid metabolism | 2 | | | |
| protein metabolism | 30 | | | |
| amino acid derivative metabolism | 2 | | | |
| one-carbon compound metabolism | 2 | | | |
| purine nucleotide metabolism | 2 | | | |
| purine nucleoside triphosphate metabolism | 2 | | | |
| nucleoside triphosphate metabolism | 2 | | | |
| steroid metabolism | 2 | | | |
| heterocycle metabolism | 2 | | | |
| purine ribonucleoside triphosphate metabolism | 2 | | | |
| glutathione metabolism | 2 | | | |
| L-serine metabolism | 2 | | | |
| glycogen metabolism | 2 | | | |
| ribonucleoside triphosphate metabolism | 2 | | | |
| ATP metabolism | 2 | | | |
| carbohydrate metabolism | 14 | | | |
| sulfur amino acid metabolism | 2 | | | |
| purine ribonucleotide metabolism | 2 | | | |
| nucleoside phosphate metabolism | 2 | | | |

Table 10

# Method Comparison

## Representative Expression of Liver Function Proteins

| General Homeostasis | Liquid Tissue Prep | Wang Prep Liquid Fraction | Wang Prep DNA Fraction | Banerjee Prep |
|---|---|---|---|---|
| glucose homeostasis | 1 | | | |
| cell glucose homeostasis | 1 | | | |
| hydrogen ion homeostasis | 1 | | | |
| monovalent inorganic cation homeostasis | 1 | | | |
| cell ion homeostasis | 1 | | | |
| cation homeostasis | 1 | | | |
| ion homeostasis | 1 | | | |
| homeostasis | 2 | | | |
| cell homeostasis | 2 | | | |
| **Vitamin Utilization and Storage** | | | | |
| water-soluble vitamin metabolism | 3 | | | |
| vitamin metabolism | 3 | | | |
| vitamin B6 metabolism | 1 | | | |
| vitamin binding | 2 | | | |
| **Fat Metabolism** | | | | |
| fatty acid beta-oxidation | 1 | | | |
| protein amino acid sulfation | 1 | | | |
| fatty acid oxidation | 1 | | | |
| fatty acid metabolism | 4 | | | |
| fatty acid transport | 1 | | | |
| **Bile Production** | | | | |
| bile acid metabolism | 1 | | | |
| **Iron Storage and Utilization** | | | | |
| iron-sulfur cluster assembly | 1 | | | |
| iron ion binding | 2 | | | |
| **Ammonia Conversion** | | | | |
| threonine ammonia-lyase activity | 1 | | | |
| L-serine ammonia-lyase activity | 1 | | | |
| ammonia-lyase activity | 1 | | | |

Table 11

# Method Comparison

## Representative Expression of Liver Function Proteins

| | Liquid Tissue Prep | Wang Prep Liquid Fraction | Wang Prep DNA Fraction | Banerjee Prep |
|---|---|---|---|---|
| Total Proteins Involved in Liver Function | 677 | 10 | 3 | 2 |
| % of Total Proteins Identified Involved in Liver Function | 677/1251<br>54% | 10/107<br>9% | 3/12<br>25% | 2/15<br>13% |

Table 12

# Method Comparison

**Representative Expression of Liver Function Proteins**

| Proteins Associated with Liver Tissue | Liquid Tissue Prep | Wang Prep Liquid Fraction | Wang Prep DNA Fraction | Banerjee Prep |
|---|---|---|---|---|
| Alanine aminotransferase (ALT) | yes | no | no | no |
| Alkaline phosphatase (ALP) | yes | no | no | no |
| Aspartate aminotransferase | yes | no | no | no |
| Albumin | yes | no | no | no |

- Alanine aminotransferase (ALT) is an enzyme present in hepatocytes (liver cells)
- Alkaline phosphatase (ALP) is an enzyme in the cells lining the biliary ducts of the liver.
- Aspartate aminotransferase is an enzyme associated with liver parenchymal cells.
- Albumin is a protein made specifically by the liver.

Figure 11

# Liquid Tissue Patent

**US Patent Application 10/769,288**

Claim 1:

A method of preparing a biomolecule lysate, comprising the steps of:

(a) heating a composition comprising a histopathologically processed biological sample and a reaction buffer at a temperature and a time sufficient to reverse of release protein cross-linking in said biological sample, and

(b) treating the resulting composition with an effective amount of a proteolytic enzyme for a time sufficient to disrupt the tissue and cellular structure of said biological sample, wherein said biomolecule lysate is in a *soluble liquid form suitable for protein expression analysis* and wherein the content of said *lysate is representative of the total protein content* of said histopathologically processed biological sample.